REMARKS

Claims 37-61 are pending, upon entry of the amendment submitted above. Favorable reconsideration is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, second paragraph, is believed to be obviated by the amendments submitted above. In re-writing the claims, the issues raised in the Official Action are believed to have been addressed. Therefore, the claims are definite within the meaning of 35 U.S.C. §112, second paragraph. Accordingly, withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, first paragraph, deposit requirement, is believed to be obviated by the executed Declaration from Dr. Mock enclosed herewith. Withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, first paragraph, enablement, is believed to be obviated by the amendments submitted above. The specification teaches how to make and use the claimed compositions. Accordingly, withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §103(a) over Kraevets et al., Derwent abstract only, is respectfully traversed. Kraevets et al. fail to suggest the claimed compositions.

Kraevets et al. disclose immunogenic compositions or vaccines containing a protective antigen and a ***live strain*** of *B. anthracis* which lacks the capsule (i.e., lack of the pX02 plasmid). The reference fails to describe the use of killed spores of *B. anthracis*.

The Examiner has asserted that:

> *Killed anthrax vaccines were well known in the prior art to be safe and efficacious when used in humans*... it would have been obvious to substitute killed spores of a non-encapsulated strain of *B. anthracis* in place of the live spores taught by Kraevts. [See the Official Action dated November 3, 2004 at page 10.]

However, the Office has cited no reference or other evidence to support that assertion. In fact, ***killed spores of B. anthracis have not been used in the development of an anthrax vaccine in the art.***

Applicants submit herewith excerpts from a book entitled The Anthrax Vaccine: Is it Safe? Does it Work?". Referring to chapter 2, entitled "Background," it emerges that there were no attempts to develop killed anthrax vaccines in the art.

More specifically, pages 48-49 describe the development of anthrax vaccine. The main directions for the development are:

(1) a heat attenuated vaccine,

(2) a live spore vaccine developed by Sterne,

(3) non-living vaccines, which correspond to protective antigen (PA) (see Turnbull, 2000 and Turnbull, 2001 enclosed herewith):

a. the US vaccine (cell-free culture filtrate of V 770-NP1-R strain) is based on cultures on synthetic medium (528 medium) without proteins or other macromolecules,

b. the UK vaccine (cell-free culture filtrate), a protein hydrolysate was preferred to synthetic 528 medium and Sterne strain 34F2 was adopted (see Turnbull, 2000 enclosed herewith).

Applicants also submit herewith a copy of a publication co-authored by Dr. Mock, the inventor of the present application: "Anthrax Spores Make an Essential Contribution to

Vaccine Efficacy," *Infection and Immunity*, February 2002, pp. 661-664. This article was published after the effective U.S. filing date of the present application. This paper describes that inactivated (i.e., killed) spores of *B. anthracis* induced a protective effect (see the Abstract). The publication concludes with the following paragraph:

> In summary, we present evidence that inclusion of killed spores greatly enhances the protective efficacy of a PA-based vaccine. Immunization with FIS plus PA provides a synergistic protective immunity acting on both toxemia and infection. The immune response induced by FIS may act early by blocking germination, a critical step at the onset of pathogen multiplication (6, 31). The molecular mechanisms of the protective immunity induced by FIS and the putative role of spore antigens need to be further investigated. It is clear that full protection against anthrax requires a multifactorial immune response. ***The results presented here may serve as the basis for the first design, for human use, of a subunit vaccine as protective as the current live veterinary vaccine***. [Emphasis added.]

Thus, this publication from Dr. Mock establishes that killed spores for use as an anthrax vaccine were not known prior to the present invention.

In view of the foregoing, one of ordinary skill in the art would not have been motivated to replace live spores with killed spores, considering that the easiest possible manipulation is antigen manipulation. Therefore, the claimed composition are not suggested by Kraevets et al.

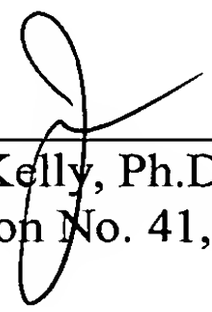
In addition, the experimental data presented in the specification is striking evidence of non-obviousness. The data in Tables I, II and III at pages 12, 13 and 15 of the specification demonstrate that the claimed compositions and vaccines provide complete protection and have the same efficacy as the live vaccine. These results are unexpected from Kraevets et al., since that reference describes the use of live spores only.

In view of the foregoing, the claims are not obvious over Kraevets et al. Accordingly, withdrawal of this ground of rejection is respectfully requested

Applicants submit that the application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.
Norman F. Oblon



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DOCKET NO: 220572US0X PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

MICHELE MOCK :

SERIAL NO: 10/069,961 : EXAMINER: J. E. GRASER

FILED: JULY 19, 2002 :

: GROUP ART UNIT: 1645

FOR: ACELLULAR IMMUNOGENIC COMPOSITIONS AND ACELLULAR VACCINE COMPOSITIONS AGAINST BACILLUS ANTHRACIS

DECLARATION

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

Now comes Michelle Mock who deposes and states that:

(1) I am the inventor of the above-identified application.

(2) The strains RPLC2 (Collection Nationale de Cultures et de Microorganismes held by the Institute Pasteur (28 rue du Dr Roux, 75724 Paris Cedex 15, France) under the number I-2270, dated July 28, 1999) and RP42 (Collection Nationale de Cultures et de Microoganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999) have been deposited under the terms of the Budapest Treaty. I confirm that the deposit has been accepted by an International Depository Authority under the Treaty, that all restrictions upon public access to the deposited material will be removed upon the grant of a patent from the present application, and that the deposited materials will be replaced if viable samples of the material cannot be dispensed by the depository.

(3) The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

(4) Further deponent saith not.

May 2 2005
Michele Mock

Date

Annex 1

The Anthrax Vaccine: Is It Safe? Does It Work?

Lois M. Joellenbeck, Lee L. Zwanziger, Jane S. Durch, and Brian L. Strom, *Editors*, Committee to Assess the Safety and Efficacy of the Anthrax Vaccine, Medical Follow-Up Agency

288 pages, 6 x 9, 2002

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Description

The vaccine used to protect humans against the anthrax disease, called Anthrax Vaccine Adsorbed (AVA), was licensed in 1970. It was initially used to protect people who might be exposed to anthrax where they worked, such as veterinarians and textile plant workers who process animal hair. When the U. S. military began to administer the vaccine, then extended a plan for the mandatory vaccination of all U. S. service members, some raised concerns about the safety and efficacy of AVA and the manufacture of the vaccine. In response to these and other concerns, Congress directed the Department of Defense to support an independent examination of AVA.

The Anthrax Vaccine: Is It Safe? Does It Work? reports the study s conclusion that the vaccine is acceptably safe and effective in protecting humans against anthrax. The book also includes a description of advances needed in main areas: improving the way the vaccine is now used, expanding surveillance efforts to detect side effects from its use, and developing a better vaccine.

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Background

Anthrax is primarily a disease of animals, and historically, humans have generally contracted the disease through contact with infected animals or contaminated animal products. The disease had become extremely uncommon in any form in the United States until the intentional mailings of anthrax spores caused an outbreak in the autumn of 2001 that resulted in five deaths from the inhalational form of the disease.

Anthrax vaccines for use in animals were first developed in the late 19th century. Work on vaccines suitable for human use gained urgency in the 1940s, with fears that anthrax would be used as a biological warfare agent. The current vaccine, Anthrax Vaccine Adsorbed (AVA), was licensed in 1970 and was recommended for use by a small population of textile mill workers, veterinarians, laboratory scientists, and other workers with occupational risk of exposure to anthrax. In the 1990s, increased concern about the use of biological weapons led the Department of Defense (DoD) to begin vaccination of U.S. military personnel. Some troops were given anthrax vaccine in the 1991 Gulf War, and a large program to vaccinate all service members was begun in 1998. By 2001 a limited vaccine supply, the result of delays in federal approval for release of newly manufactured vaccine lots, had significantly slowed plans to vaccinate all military personnel. After the deliberate distribution of anthrax spores in bioterrorist incidents in the autumn of 2001, the vaccine was offered as part of the treatment for as many as 10,000 of the civilians who had been exposed.

This chapter summarizes the basic pathophysiology of anthrax, reviews the history of anthrax vaccine development, and outlines the concerns that

have emerged on the part of some people about the adverse health outcomes that might be associated with use of the vaccine.

THE DISEASE

Anthrax is caused by infection with *Bacillus anthracis*, a gram-positive, nonmotile, spore-forming organism (Brachman and Friedlander, 1999; Dixon et al., 1999). Exposure to the spores of this one organism can cause three different forms of disease—cutaneous, gastrointestinal, or inhalational anthrax—depending on the site of infection. Cutaneous anthrax is the most common and the most treatable form; inhalational anthrax is rare but poses a much greater risk of death.

Epidemiology

Anthrax is found worldwide and is transmitted primarily through spores that are highly resistant to heat, drought, and many disinfectants (Dixon et al., 1999). It is primarily a disease of wild and domestic animals, especially herbivores such as cattle, sheep, and goats. Animals can be infected through exposure to spores in contaminated grazing areas, contaminated feed, or infected carcasses (Friedlander, 2000). Humans in agricultural settings can be infected through contact with infected animals or contaminated animal products. Human infections also occur in industrial settings where contaminated animal products such as wool, hair, hides, or meat are processed. Most human cases in either agricultural or industrial settings are cutaneous. Inhalational anthrax is generally seen only in industrial settings because conditions where a sufficiently large number of spores are aerosolized in an enclosed area do not generally occur naturally (Brachman and Friedlander, 1999). Person-to-person transmission is not known to occur with inhalational anthrax and has rarely been reported with other forms of the disease (Friedlander, 2000).

The worldwide incidence of anthrax in humans is difficult to determine, but the annual number of cases in the 1980s and 1990s is estimated to have been about 2,000, down from an estimated 20,000 to 100,000 cases in 1958 (Brachman and Friedlander, 1999). During the 19th century, "wool-sorters' disease," or inhalational anthrax, was fairly common among workers handling animal hides, hairs, or wools. Approximately 200 cases were reported in the United States before 1900 (Plotkin et al., 1960). Only 18 cases of inhalational anthrax were reported in the United States in the 20th century, despite evidence of extensive exposure of workers in goat hair-processing mills to aerosolized spores (Inglesby et al., 1999). In 1957, five cases of inhalational anthrax, four of them fatal, occurred at a goat hair-processing mill in New Hampshire. Vaccination did not become man-

datory for these workers until the 1960s and so cannot account for the low rate of inhalational disease (Inglesby et al., 1999).

Other forms of anthrax are also rare in the United States. Until the bioterrorist events in the autumn of 2001, a total of 238 anthrax cases had been reported since 1955; of those, 95 percent were cutaneous infections (Brachman and Friedlander, 1999; CDC, 2001a). In 2000, one reported case of cutaneous anthrax occurred (CDC, 2001a) and possible cases of gastrointestinal infection were associated with the consumption of contaminated meat (CDC, 2000b).

In the autumn of 2001 the United States experienced an outbreak of anthrax due to bioterrorism. Exposure to letters containing *B. anthracis* spores sent through the U.S. mail resulted in seven confirmed and five suspected cutaneous cases and 11 confirmed inhalational cases (CDC, 2001e). The victims included postal workers (Gallagher and Strober, 2001), employees of print and broadcast media organizations, and at least one infant (Roche et al., 2001).

Anthrax has also been part of biological warfare programs in some countries. In 1979 in Sverdlovsk, Russia, an apparently accidental release of aerosolized spores from a military facility resulted in 68 deaths among 79 individuals with reported cases of inhalational anthrax (Meselson et al., 1994).

Clinical Features

The outbreak of inhalational and cutaneous anthrax in the United States during the autumn of 2001 produced far more clinical and public health experience with the disease than had occurred in many decades. Both the outbreak and the outcomes of individual cases showed considerable differences from previous classic descriptions. The anthrax spores appeared to have been processed intentionally to enhance their most dangerous properties. They were finely milled and rendered nonpolar to maintain the very small particle size necessary for inhalation and to promote prolonged aerosolization. Naturally occurring spores tend to adhere quickly to each other and to surfaces.

Improvements in both the speed of diagnosis and clinical management resulted in the survival of at least some of those who contracted inhalational anthrax, which would not have been expected on the basis of earlier experience (Brown, 2001). Analysis of this new information on the clinical course of disease was continuing as the committee completed this report. In particular, both the inoculum associated with infection in different individuals and the duration of antibiotic treatment necessary for survival after infection remain uncertain. Although every effort was made to include

been renovating biological, chemical, and nuclear warfare research sites 3 years after barring international inspectors (Bohlen, 2001). In 1997, as a result of the concerns about biological weapons, then Secretary of Defense William Cohen initiated a plan to vaccinate all U.S. service members against anthrax. Immunizations began in March 1998 under DoD's Anthrax Vaccine Immunization Program (AVIP). As of November 29, 2001, 522,529 service members had received 2,098,544 of doses of AVA (http://www.anthrax.osd.mil/Flash_interface/default.html, accessed January 11, 2002).

Implementation of AVIP has been slowed by a limited supply of vaccine. Renovations were begun at the manufacturing plant in 1998, and BioPort, the sole manufacturer, did not receive approval from the Food and Drug Administration (FDA) for release of newly manufactured vaccine until January 31, 2002. DoD has been able to continue immunizations, despite the limited supply of vaccine, but not at the rate first planned. In July 2000, in November 2000, and again in June 2001, DoD slowed the anthrax immunization program, focusing only on troops thought to be at greatest potential risk (http://www.anthrax.osd.mil, accessed September 5, 2000; Marshall, 2000).

In the autumn of 2001, more than 30,000 civilians were potentially exposed to anthrax in bioterrorist incidents involving the distribution of highly infectious spores through the U.S. mail (CDC, 2001d,e). Beginning in December 2001, CDC began offering vaccination with AVA as a treatment option for selected exposed civilians. This therapeutic use of the vaccine following exposure was not included under the official vaccine license and is being monitored under the provisions of an Investigational New Drug application. As of February 25, 2002, the latest data available at the time that this report was completed, 192 people had begun receiving doses of AVA (Ashford, 2002).

The committee emphasizes that this report is addressed to DoD and focuses on the licensed use of AVA for immunization before exposure to anthrax spores.

CONCERNS ABOUT USE OF AVA

AVIP and the product AVA have become focal points of great concern on the part of at least segments of the military and interested public. A few service members have refused the vaccine, at the risk of court-martial, because of their perception that it is particularly dangerous.[2] Among the

[2]Studies of service member's knowledge, attitudes, and beliefs regarding the anthrax vaccine are being planned by CDC to help DoD to better understand and respond to such concerns.

concerns are complaints among Gulf War veterans of chronic multisystem clinical conditions that still lack a definable relationship to the anthrax vaccine or to other events in their Gulf War experiences (IOM, 2000b).

The U.S. Congress has responded to these concerns, and hearings have been held in the U.S. House of Representatives and the U.S. Senate.[3] The hearings typically included several current or former service members (or family members of service personnel) who had raised concerns about the adverse events that they had experienced or observed or about the responses of military health care providers to these concerns. The witness list usually also included officials from DoD or a branch of the military service, FDA, and sometimes the manufacturer of AVA. As noted in Appendix C, the IOM Committee to Assess the Safety and Efficacy of the Anthrax Vaccine also held a public hearing to gather information from people with concerns about the vaccine. The IOM committee benefited from the perspective provided by the speakers, many of whom also provided testimony during congressional hearings. The witnesses described persistent and debilitating symptoms ranging from fever, headache, and malaise to swelling, joint pain, and tinnitus, which they ascribed to the anthrax vaccine. Several witnesses also described specific serious conditions including hypogonadism; Stevens-Johnson syndrome, which affected their vision as well as their skin; and aplastic anemia, which proved fatal. In addition, many witnesses observed that when they reported their symptoms to medical personnel, the health care providers seemed to be unaware of the Vaccine Adverse Event Reporting System (VAERS) or unwilling to file a report with VAERS and often seemed to doubt that the vaccine could have caused their symptoms.

AVAILABLE DATA ON AVA

In its letter report of March 2000, *An Assessment of the Safety of the Anthrax Vaccine*, the IOM Committee on Health Effects Associated with Exposures During the Gulf War expressed regret over the lack of information about the vaccine in the peer-reviewed published literature (IOM,

[3]Christopher Shays, chair of the Subcommittee on National Security, Veterans Affairs, and International Relations of the Committee on Government Reform and Oversight convened a series of hearings in 1999 and 2000 on AVIP and on allegations that adverse event reporting to the Vaccine Adverse Event Reporting System does not adequately reflect the actual rate of adverse events. Congressman Steve Buyer, chair of the Subcommittee on Military Personnel of the House Committee on Armed Services, also held a hearing on AVIP, and the Committee on Appropriations chaired by Ted Stevens held a hearing on Gulf War illnesses, as had Congressman Shays. The Senate Committee on Armed Services, chaired by Senator John Warner, also held 3 days of hearings in 2000. Those hearings were on AVIP and DoD's antibiological warfare agent vaccine acquisition program.

2000a). It listed an array of studies that were unpublished or ongoing that could contribute to the body of information on which conclusions regarding health effects could be based.

As the study presented in this report began, representatives of DoD provided assurances to IOM that all relevant information from DoD would be made readily available to the committee and that efforts would be made to publish the data from completed studies. DoD and its investigators have followed through on these assurances. Most of the studies that have been carried out by DoD investigators to assess the safety and efficacy of the anthrax vaccine have now been written up as manuscripts and submitted for publication. Additional studies have been published since the letter report was released (e.g., CDC, 2000a; Gunzenhauser et al., 2001; Pittman et al., 2001, 2002; Rehme et al., 2002). In addition, one of the most important contributions to the committee's evaluation was in the form of analyses of data from military databases carried out at the committee's request. In accordance with its charge, the earlier IOM committee (Committee on Health Effects Associated with Exposures During the Gulf War) reviewed only the published, peer-reviewed literature to reach its conclusions about safety. The current committee had a different purpose and as a result chose to review all the studies it was aware of and for which adequate descriptions of the study methods, data analyses, and results were made available. These studies are systematically reviewed in the chapters that follow.

Several previous IOM committees evaluating possible causal associations between vaccines or other exposures and specific health outcomes have chosen to describe their findings with a weight-of-evidence approach (IOM, 1991, 1994, 2000b). Their findings placed associations between the exposure of interest and the health outcome into categories such as sufficient evidence of a causal relationship, sufficient evidence of an association, limited or suggestive evidence of an association, inadequate or insufficient evidence to determine whether an association does or does not exist, and limited or suggestive evidence of no association. The current committee chose not to use that approach because it was not asked to evaluate exposure to AVA as a cause of specific health outcomes. Rather, the committee was asked to provide an overall evaluation of the anthrax vaccine's safety. In addition, its charge included addressing various aspects of the efficacy of AVA, as well as manufacturing issues, two topics for which a weight-of-evidence approach is not readily applicable.

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Annex 3

Current status of immunization against anthrax: old vaccines may be here to stay for a while

Peter C.B. Turnbull

Anthrax vaccination has become a 'hot' topic. On the one hand, fears that Iraq holds secret caches of anthrax-based weaponry, that other countries may be developing or may have developed similar devices, or that hard-line groups may make their own anthrax-based devices for bioterrorist attacks have focused official attention on the need for means of protection, principally, though, for the military. On the other hand, the unsolved issues of the Gulf War illnesses have left elements of doubt in the minds of some as to the possible role of anthrax (among other) vaccines in this syndrome, and have drawn attention to the shortage of pre-clinical, clinical, pharmacological and safety data on the existing UK and US anthrax vaccines. In the middle are those hotly debating the US and Canadian policies of mandatory anthrax immunization for military personnel or, in the case of the UK policy of voluntary immunization, simply voting with their feet. Compounding matters have been the publicized failures of the US vaccine production facility and the less publicized UK problems of supply. Meanwhile, those in genuine at-risk occupations are left unsure whether, if they can get the vaccine at all, they really want it. Despite two decades of elegant science aimed at formulating alternative vaccines to overcome all the problems of efficacy, safety and supply, such an alternative is at least five years away, and the current status is that we must live with the old vaccines or not vaccinate. Curr Opin Infect Dis 13:113-120. © 2000 Lippincott Williams & Wilkins.

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Abbreviations

BW	biological warfare
DoD	US Department of Defense
GWI	Gulf War illness
PA	protective antigen
Th	T helper

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Introduction

The proofs of what ultimately became quite a well-received review on anthrax vaccines [1] arrived just as the build-up to the Gulf War was beginning, and, to the consternation of the journal editor, the article was directed into cold storage by order from on high. The offending statement, which the relevant authorities felt might not inspire the appropriate levels of confidence in allied fighting forces, was: '... tests in animals have indicated that the protective efficacies of both the UK and US vaccines are less than ideal'.

Now, some nine years later, interest in the topic of immunization against anthrax remains integrally associated with the concept that, of the agents that might be used for biological warfare (generally reduced to the less inflammatory 'BW') or bioterrorism (euphemistically called 'Deliberate Release' in the UK), anthrax and smallpox pose by far the greatest threats [2,3]. Enhancing this interest are the unsolved issues of the so-called Gulf War syndrome.

Also heightening interest has been the increasing influence of the rapid communication channels, particularly ProMED, and reference to these generally non-citable sources is unavoidable for an effective review of this topic. Compounding the situation has been the revelation on 'the web' and elsewhere that the US facilities for producing the US anthrax vaccine had been failing for some time to meet Food and Drug Administration production standards [4–6]. Although it is not on the web or otherwise publicized, practitioners and others who have wanted to obtain the UK vaccine for at-risk occupational needs over the past two years have noted the existence of problems here also.

If these factors and situations are seen metaphorically as a gasoline-soaked bonfire, then the match that turned this into an inferno was the US Department of Defense's (DoD) DoD-wide anthrax immunization directive issued by Defense Secretary William S. Cohen in December 1997 [7,8••,9,10]. Under this, the full course of anthrax vaccination, some six doses over an 18 month period, became mandatory for all serving US military personnel. Pro- and anti-propaganda raged in the formal and informal media and the requirement remains a major bone of contention. It seems that Canada adopted a similar mandatory policy for its troops [3,11]. The UK Ministry of Defence watched these developments with

interest and came up with a non-mandatory alternative [3]. In that there is often not a lot of enthusiasm on the part of vaccinees for successive doses, this approach has its own inherent problems [12,13].

On the sidelines to all of this are the individuals in at-risk civilian occupations, whose fear of the disease has increased with the hype about BW and bioterrorism while, at the same time, having their concerns about vaccination raised.

The remainder of this review attempts to put the present and future of anthrax immunization into a balanced context.

Current vaccines

In its natural state, anthrax is primarily a disease of wild or domestic herbivorous animals. Humans are incidental hosts and, with the exception of rare cases in the past of laboratory-acquired infection, almost invariably contract the disease directly or indirectly from animals that have died of it. It has long been recognized that the best method of control of anthrax in humans is control in livestock; fundamental to control in livestock is vaccination and a well known landmark in history was Pasteur's anthrax vaccine [14]. Although still used today in Italy, Pasteur's vaccine, based on capsulating non-toxigenic mutant derivatives of virulent *Bacillus anthracis*, was largely supplanted by the toxigenic, non-capsulating strain 34F2 live spore vaccine of Sterne, developed in the 1930s [15] or by its analogues used in countries of the former USSR and in China.

The animal vaccines, although highly successful in reducing anthrax from a major global problem to a relatively minor, but ongoing, nuisance, have always been associated with a residual virulence; occasional casualties among animals receiving them led to them being regarded as unsuitable for human use in the West (although human equivalents are used in China and the former USSR). It was military interest after World War II in the potential use of anthrax as a BW agent that resulted in the extensive work at Porton Down in the UK and Fort Detrick in the USA in the 1950s to develop non-living vaccines.

Despite good communication between the UK and US groups, the emergent vaccines, although analogous in many ways, did develop along slightly different routes. The US vaccine, from the start, was based on cultures in synthetic medium without proteins or other macromolecules. Initially, '528 medium' was used [16]. Alum precipitation of the filtrate was found to confer stability and enhance the immunizing potential in mice [16] and to induce 'effective immunity' in rabbits, guinea pigs and monkeys, as well as being well tolerated by human volunteers [17]. Anaerobic incubation as a procedural modification to simplify large-scale production was adopted [18] and the 528 medium modified, via 599 medium [19], to 1095 medium [20,21]. However, it was found that protective antigen (PA) in the anaerobic cultures was not readily precipitated by alum, and adsorption onto aluminium hydroxide gel (alhydrogel) was adopted instead [22]. At this point the vaccine was virtually as described in Patent 3,208,909 [23], but the strain of *B. anthracis* to be used had not been decided. Ultimately, strain V770-NP1-R, a rough, non-encapsulated, non-proteolytic variant of a bovine isolate in Florida in 1951 [24], was adopted and today's US vaccine (product licence no. 99) is an alhydrogel-adsorbed cell-free culture filtrate of V770-NP1-R, grown anaerobically in a fermenter. It was licensed in 1972 for administration to those in at-risk occupations [25]; it is not licensed for use in children or pregnant women [26] but, at present, this vaccine is not available for civilian use anyhow [2,27].

The UK vaccine (licence numbers 1511/0037 and 0058), made today essentially as first formulated in 1954 [28], went through a relatively simple evolution, diverging immediately from the Wright and colleagues [16–18,20–24] vaccine in that a protein hydrolysate was preferred to synthetic 528 medium and Sterne strain 34F2 was adopted from the outset. The aerobic static culture in 500 ml volumes of media with alum precipitation of the culture filtrate remained in place. The vaccine was introduced for workers in at-risk occupations in 1965 [29] and licensed for human use in 1979 after biological agents first fell under the European Directive 75\319\EEC.

In the former USSR, the potential of BW was again the driving force behind the development of their live spore vaccine for humans in the 1930s and 1940s [30], using strain STI-1, analogous in its derivation to Sterne's 34F2. It was licensed for administration by scarification in 1953 and by subcutaneous injection in 1959. An analogous live vaccine is used in China [31,32]. To the author's knowledge, other countries have not concerned themselves with the need to produce a human anthrax vaccine, and seemingly, for the small number of doses needed for the few persons in seriously at-risk occupations (principally laboratory workers), have relied on being able to obtain the US or UK vaccines.

Efficacy of current vaccines

Human data

The need to revisit this history for a 'current status' article lies in the fact that the ongoing debates alluded to in the Introduction revolve around the clinical evidence of efficacy in humans and of the safety of the existing UK and US vaccines. Because target populations for

clinical trials are now virtually impossible to identify for human anthrax, and volunteer tests are out of the question, much is made of the information obtained when the US vaccine was first introduced into mills processing animal products from anthrax-endemic areas of the world [19]. In a comparison of mill workers who had received the vaccine and those who had not, a 92.5% degree of effectiveness against the cutaneous form of anthrax was found among those who had had the full course of six doses; four cases occurred in persons who had received incomplete courses, but one occurred in a fully vaccinated person. The problem in viewing these data today, however, is that the vaccine used was an alum-precipitated culture filtrate of strain R1-NP (of totally different origin to V770-NP1-R) in 599 medium. Filter-sterilization procedures changed in 1990, which may also have altered the final product [33]. The preservative in the original vaccine, thiomersal, has been changed to benzathonium chloride in the current vaccine, and formalin, now included as a stabilizer, was not present in the original vaccine. The vaccine assessed in the mill workers [19] therefore differed somewhat from the licensed vaccine in use now [6,8••,34,35], and no equivalent trials were performed with the present vaccine [6,36]. However, no cases of anthrax have been recorded in individuals who have received two doses of the current vaccine with seven or more days elapsing since the last dose or three doses, regardless of the time elapsed since the third dose [35].

No such studies were carried out on the UK vaccine. Its apparent value was confined to observations that the incidence of anthrax was decreasing in the wool and hide industries where vaccination programmes were in place, whereas infection rates outside these trades, e.g. among gardening enthusiasts, remained steady [37], and that the number of reported cases declined fourfold between 1961–65 and 1976–80, corresponding in time with the introduction of the vaccine [38]. There is also no case on record of anthrax in a human vaccinated with the UK vaccine. However, there are, of course, a number of other factors to which the declines in anthrax could be attributed, and similar declines were occurring in other countries of Europe and Scandinavia that did not use a human vaccine.

In the case of the STI-1 vaccine in the former USSR, good target populations for clinical trials existed in the southern European and middle Asian Soviet republics, where a 75–84.2% rate of effectiveness was apparently recorded [36].

Animal studies

By and large the efficacy of the UK and US vaccines has been judged on the results in animal studies, initially those cited above associated with the design of the vaccine, but later those carried out in the 1980s after the Sverdlovsk incident [39] had re-awakened concern about the possible use of anthrax as a BW agent. Returning to the 'offending statement' in the opening paragraph of this article, the majority of the protection studies carried out in the 1940s to 1960s involved challenge with the Vollum strain, or derivatives of it, a strain of uncertain origin except that it was isolated from a cow in Oxfordshire before World War II. In the 1980s, it became clear in studies, mostly with guinea pigs (chosen as being both highly susceptible and convenient), that the Vollum strain was unusually acquiescent to the protective effects of the vaccines and, when a range of other challenge strains was used, the performances of the vaccines were somewhat less impressive [1,40].

In the aftermath of the Gulf War and with continued concern that anthrax could be the weapon of warfare or terrorism, further studies were carried out, this time concentrating on the animal model in which protection was being tested. The interesting conclusion has been that the species in which efficacy is being evaluated is very important; in rhesus macaques [41,42•] and rabbits [43] the US vaccine appears to be able to confer a high degree of protection against the Ames strain of *B. anthracis*, which is among those that appear to overcome vaccine-induced immunity in guinea pigs quite readily.

Real and perceived problems with the current vaccines

Both the UK and the US vaccines are produced and administered under what is essentially a 'licence of right' as a result of being in existence for so long. The pre-clinical, clinical, pharmacological and safety data that would be required for a new product to be licensed today were never generated. The production processes and efficacy testing procedures (acceptance criteria are based entirely on animal potency and toxicity tests) make batch-to-batch variation difficult to control or monitor effectively, and this is made harder by the constraints of biohazard level 3 (ACDP 3) containment under which they have to be produced. The contents of the final product are poorly defined, being essentially adsorbed or precipitated culture filtrates and, as a result, there are no precise ways of determining their shelf-life. Based on culture in protein-based media, the UK vaccine is subject to regulations relating to ensuring the absence of bovine spongiform encephalopathy. Even the rationale behind the complex dose schedules (six and five doses over 18 months for the US and UK vaccines, respectively) are lost in history, differing from those used in the primate tests by which the vaccines were originally judged efficacious.

The vaccines are also associated with complaints of reactogenicity. Although this rarely exceeds mild

erythema and soreness and swelling at the site of injection lasting 2 or 3 days. this is a primary reason for objections by members of the US (and Canadian) armed forces to the mandatory vaccination policy [7,11]. The package insert of the US vaccine cites a 30% rate of mild local reactions and a 4% rate of moderate local reactions with a second dose, and medical monitoring of over a million military personnel deployed recently in the Gulf region found short-term effects commensurate with this [44]. In a voluntary programme, such as that offered by the UK Ministry of Defence [45], reactogenicity is a major reason why relatively few recipients will receive the full immunization course [12]. The safety of the vaccines, however, was never seriously questioned [6], and even those basically opposing the decision to vaccinate all US armed forces personnel have not really attempted to do so on the grounds of safety, beyond the fact that the published safety data are limited [8••,46,47]; this was officially addressed [48–50]. Other clinical reasons for opposition have been the lack of efficacy data in primate tests against a wide enough range of challenge strains [51], although a broader list of reasons why a vaccine against anthrax might not be particularly useful in the context of aggressive use can be drawn up [8••].

It is an observation in passing that 'in more than 30 years' experience no instances of adverse effects following the use of the (Russian) STI human vaccine were recorded' [30]. No information was found on the Chinese equivalent.

Gulf War Illnesses

Although 'some Gulf War veterans are suffering from unexplained illness they believe might have been caused by anthrax vaccines that they received during the war' [6], and fear of the development of Gulf War illness (GWI) is recorded as a reason for the poor uptake of offered vaccine among British military personnel [13], the US Presidential Advisory Committee on Gulf War Veterans' Illness concluded that 'it is unlikely that health effects reported by Gulf War veterans today are the result of exposures to ... anthrax vaccines, used alone or in combination' [52]. It was the US General Accounting Office's assessment [52] that the Department of Defense did not adequately monitor the effects of receiving multiple vaccinations, but overall, the voluminous documentation on GWI has not bent heavily towards incriminating anthrax vaccine *per se* in this syndrome. However, according to Nass [8••], questions persist regarding its role in the development of GWI.

Despite regular updates on the web (http://www.mod.uk/policy/gulfwar/index.htm), the UK government has yet to issue its conclusions on the role, if any, of vaccines in GWI. However, much has been made of the combined use of the UK vaccine with the whole cell pertussis vaccine [8••,53•], and similarly of suspicions, denied by the DoD [54], that unlicensed anthrax vaccine formulations with squalene-based adjuvants, shown in animal tests to give good protection, had been given to US soldiers at the time of the Gulf War. This revolved around claims of anti-squalene antibodies in many of the patients [8••,53•,54]. It was also focused on in the popular literature [55]. The scientific basis for the possible adverse effect of the confirmed UK/pertussis combination or the putative US/squalene formulation is put down to a shift in cytokine balance from T helper (Th) type 1 to Th2 cells (see below) as a result of the novel additions [8••,56].

New vaccines?

Concerns that arose in the 1980s after the Sverdlovsk incident about the efficacy of the existing human vaccines in the West, and the GWI debate and opposition to mandatory armed forces vaccination this past decade have focused attention on the lack of pre-clinical, clinical, pharmacological and safety data on these vaccines. Considerable funding has gone into research directly or indirectly concerned with developing next-generation vaccines that would meet the current production, efficacy and safety criteria, as well as having improved performance characteristics.

From the production standpoint, the new vaccine needs to be fully defined with quantitative methods of analysing the components, clear standardization, and a production method that ensures batch-to-batch reproducibility. With this will come the means of generating the desired pharmacological data (rate of distribution in the body, half-life, immune response details, etc.), safety data and data on shelf-life. In addition, it is frequently claimed that the reactogenicity associated with anthrax vaccines will be reduced or eliminated in vaccines lacking the non-active ingredients inevitably present in the current versions. Further targets are to obtain unequivocal efficacy in a shorter time period and with a much simpler dose regime than current regimes, the ultimate being a single orally administered dose, and that it should be part of a single vaccine against multiple agents. Finally, of course, it should be cheap.

As reviewed previously [1], the results of some intensive research in the 1980s were putative prototypes, which could be broadly divided into subunit vaccines, based on purified PA with what was then viewed as 'cell-mediated immunity-stimulating' adjuvants, recombinant vaccines in which the cloned PA gene was expressed in a non-pathogenic *Bacillus* species or virus, such as baculovirus or vaccinia, or live vaccines using highly attenuated versions of the Sterne strain of

B. anthracis. Interest in clinical trials was not forthcoming at that point [1], and subsequently the insinuations levelled at the DoD that squalene-based vaccines (anthrax vaccines implied) show that progress on the subunit vaccine proposed, which would have had a squalene component, would probably have been short-lived.

The 1990s have seen much more in-depth science on the basic immune systems behind the response to anthrax vaccines, accompanied by considerable advances in the understanding of the nature and role of the components of the anthrax toxin in the molecular pathogenesis of the disease by means of very elegant molecular studies. Accepting, as stated earlier, that there is no target population for clinical trials or other means of directly determining the efficacy of an anthrax vaccine in humans (we can dismiss [57] the slightly peculiar suggestion by one group [45] of double-blind trials), the most up-to-date knowledge and techniques in the rapidly advancing field of immunology are being applied to looking for markers of protection that would, at the end of the day, be acceptable to licensing authorities. Passive protection tests have long indicated a role for antibodies in protection [58], and progress has been made towards identifying specific isotypes that may provide markers of protection [43,59•,60].

Problems here again lie in extrapolating animal data to humans and in the increasing opposition to, and problems associated with, challenge tests in animals, particularly non-human primates, reasonably regarded as the most relevant for human needs [61]. The new knowledge that the macrophage is central to lethality in anthrax, through its toxin-mediated release from these cells of potent inflammatory cytokine mediators IL-1β and TNF-α, resulting from overload of reactive oxygen intermediates [62••,63], and further in-depth studies on the nature of lethal toxins and their action on macrophages [64–67] are providing a much clearer idea of what we are trying to protect against in prophylactic strategies. Meanwhile, one hypothesis suggests that the differences in degrees of protection afforded by the US and UK vaccines in, for example, guinea pigs and macaques, are attributable to differences in Th1 and Th2 responses in different species to antigens delivered with aluminium-based adjuvants. These adjuvants promote Th2 responses predominantly [68], but as yet, little is known about the Th1/Th2 system in guinea pigs [59•].

Favourites for the next generation vaccines are subunit vaccines, whose active ingredient is whole-length (83 000 M_r) recombinant PA, although the host organisms proposed have ranged from a modified Sterne strain [42•,69,70] or strain ANR of *B. anthracis* [71•], through *Bacillus subtilis* by means of two cloning approaches [72,73•,74] to *Escherichia coli* [75•]. Also proposed as the possible active ingredient is a mutant 63 000 M_r portion of the PA molecule, cloned in *B. subtilis*, able to induce protective immunity but unable to bind LF or EF, and therefore incapable of being toxic [76]. Avoiding the use of *B. anthracis* does away with the requirement to carry out production procedures at biohazard level 3, which places extra burdens on staff and facilities already attempting to meet increasingly stringent regulatory requirements for pharmaceutical production. In the meantime, moves towards subsequent generation vaccines are also under way with a view to the development of oral [77] and DNA vaccines [78,79]. An ever better understanding of the mechanism of the toxin at the subcellular level [80•,81] will be an aid to this. Exciting possibilities also lie ahead for the use of the anthrax toxin for vaccination against other conditions, such as cancer [82], antiviral immunity [83] or for immunotherapy [84–86].

Protective antigens

The PA component of the toxin has long been accepted as the molecule fundamental to protective immunity against anthrax, and it is well established that effective protection can be induced in various species by purified PA with a suitable adjuvant. Clarification of the universality of PA is comforting in this regard [87], although concerns were raised by the engineering of a foreign virulence factor into *B. anthracis* by Russian workers [88]. Dissection of PA to identify the active and potentially useful regions continues [64,89–91], as well as studies to elucidate its precise mode of operation [81,92–97]. Nevertheless, other possible virulence factor-based candidate antigens continue to be looked for as, among other things, possible enhancers of vaccine efficacy; examples of this are the S-layer EA1 and Sap proteins [98–101]. Developments are likely to make great strides in the foreseeable future with the completion of sequencing of the pX01 and pX02 plasmids, which carry the genes for the currently known *B. anthracis* virulence factors, the toxin and the capsule [102••], and the genome sequence is eagerly awaited in year 2000 [103••].

Veterinary anthrax vaccines

The dramatic success of the Sterne livestock vaccine, referred to earlier as turning natural anthrax from a major world problem to a minor world nuisance since it was introduced in the 1940s, has meant that there has been little interest in development work in veterinary anthrax vaccines. Nevertheless, the vaccine has a number of failings in terms of safety, virulence in certain species, the potential for environmental contamination, the limited duration of efficacy, ease of administration in wild animals and so on, and new looks at possible future modern alternatives [104] are welcome.

Conclusion

The greater proportion of this review on the 'Current status of immunization against anthrax' has dwelt on two old vaccines and their histories, compacting the elegant science of the past two years into a series of compressed paragraphs. The reason for this is that, by nature of the regulatory procedures required before a vaccine can be licensed, there is not the remotest chance that a new anthrax vaccine will be on the shelf this side of the year 2005, although the author would be as pleased as anyone if this pessimism was proved wrong. The motivating force behind the development of a new vaccine, or new vaccines, is protection against the potential aggressive use of anthrax, and is targeted, therefore, almost exclusively at troops or other selected defence personnel. However, the creation of new bioweapons could always outpace development of such new vaccines [8••] and so, in the meantime, the current situation can be summed up in the statement that 'debate will continue on the use of the old US and UK vaccines'.

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Review

Anthrax vaccines: past, present and future

Peter C.B. Turnbull

Most livestock vaccines in use throughout the world today for immunization against anthrax are derivatives of the live spore vaccine formulated by Sterne in 1937 and still use descendants of his strain 34F$_2$. Credit belongs to this formulation for effective control in many countries with considerable reduction, sometimes complete elimination, of the disease in animals and, since man generally acquires it from livestock, in man also. However, there are some contraindications of its use and situations in which it cannot be easily administered, and room for development of a successor is discussed. The human vaccines, formulated for at-risk occupations and situations, date from the 1950s (UK vaccine) and 1960s (US vaccine). The rather greater need for improvement of these as compared with the veterinary vaccine stimulated valuable research during the 1980s which has led to a number of promising candidate alternatives for the future.

EARLY LIVESTOCK VACCINE: PASTEUR, CARBOZOO AND STERNE

Some 80 years after Jenner's celebrated vaccination and publication of *An Inquiry Into the Cause and Effects of Variolae Vaccinae* (Sampson Low, London, 1798), microbiology's founding fathers had begun the first systematic studies on protection afforded by vaccination against a number of the most troublesome animal diseases of the day. Most noted of these were Pasteur's demonstrations of protective immunizations against fowl cholera in 1880[1], anthrax in 1881[2] (*Figure 1*) and rabies in 1885[3]. In fact, in the case of anthrax, close examination of the records[4] has revealed that credit for the first recorded demonstration of protection induced by attenuated strains of *Bacillus anthracis* really belonged to W.S. Greenfield at the Brown Animal Sanatory Institution in London[5,6].

It was, however, Pasteur's vaccine schedule that became adopted for use. This involved two inoculations 2 weeks apart. The first dose consisted of *B. anthracis* cells from cultures which had been incubated at 42–43°C for 15–20 days (Pasteur I vaccine) and which was pathogenic only for mice and young guinea-pigs. The second dose consisted of cells from cultures incubated at 42–43°C for only 10–12 days and which were rather less attenuated (Pasteur II vaccine).

The Pasteur duplex vaccine became widely used for cattle and sheep in Europe and South America over the next 50 years. In the 1920s and 1930s, the procedure was modified[7]. First, in the 1920s suspension of spores in 50–60% glycerine was found to increase longevity and improve the immunizing efficiency of the spores and the double Pasteurian vaccine was replaced by single vaccines consisting of spores suspended in 50% glycerol. The strains were attenuated to such an extent as to be non-virulent for rabbits but virulent for guinea-pigs and the intricate manipulations needed to meet this requirement rendered these vaccines impractical in the long run. In the 1930s, the practice of adding saponin (1–10%) to the Pasteur II or other virulent or slightly attenuated strains was introduced. Saponin at these concentrations provoked a violent inflammatory response at the inoculation site which limited generalization of the anthrax infection.

As reviewed by Sterne *et al.* (1939)[8], there was an epidemic of reports between 1929 and 1937 on the application and merits of vaccines consisting of spores from isolates designated as having various levels of virulence suspended in 4–10% saponin which was reported to neutralize the virulence. Particularly popular for a while, it seems, was 'Carbozoo', initially produced at the Istituto Sieroterapico Milanese and later in the

Figure 1 From an original monograph on anthrax vaccination entitled 'Vaccinations Preventives contre le Charbon du Betail' (Compagnie de Vulgarisation du Vaccin Charbonneux Pasteur) 1886. The operator is probably Louis Pasteur himself. (Photograph kindly supplied by Dr J. Ezzell.)

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USA by Lederle and alleged to consist of a fully virulent strain of *B. anthracis* (but which Sterne *et al.*[8] considered to be somewhat attenuated) suspended in 10% saponin. Apparently no fuller details were made public as to its constitution or manufacture although it was claimed to be almost innocuous for guinea-pigs and harmless for rabbits.

Carbozoo was evidently quite effective in terms of protection but the high saponin content appears to have resulted in unacceptable adverse reactions and presumably it was this that was predominantly responsible for its disappearance from the market. In detailed studies on this vaccine and on the influence of saponin on protection, Sterne *et al.*[8] concluded that the value of the saponin lay in the significantly enhanced protective immunity rather than the only slight reduction in virulence it produced and they 'suggested that saponin should be used with mild strains (*meaning more attenuated*) to improve immunity, rather than with strong strains to reduce virulence'. They also demonstrated that the high concentrations (10%) of saponin considered necessary by the protagonists of Carbozoo were in fact unnecessary and that 0.5% saponin was effective while being non-reactogenic.

Although effective and serving their purpose well for many years, the heat-attenuated vaccines suffered from declining potencies[9] and troublesome variations in virulence resulting in occasional untoward losses among vaccinated animals and they could not be administered safely to certain particularly susceptible species[10].

It was, in fact, Sterne's own live spore vaccine[8–12] which in the long term became the world's most potent weapon against anthrax. The strain used was a rough avirulent dissociant derived from subculture of an isolate from a case of bovine anthrax on 50% horse serum nutrient agar with incubation under a 30% CO_2 atmosphere for 24 h[10]. His final formulation consisted of 600 000–1 200 000 spores ml^{-1} suspended in 0.5% saponin in 50% glycerine–saline[12] and apart from the spore content of today's products being $\pm 10^7$ ml^{-1}, the formulation of livestock vaccines used in most countries of the world today remains essentially as specified by Sterne and still uses his strain $34F_2$.

RESIDUAL VIRULENCE IN LIVE VACCINES

Vaccine protection studies, quality control tests and reports from the field all bear witness to a residual virulence in attenuated live spore vaccines. The dose required to give a protection level against virulent challenge approaching 100% in guinea-pigs frequently results in a proportion of deaths from the immunizations[13–15] and LD_{50} values of as low as 10^3 spores were found in certain inbred strains of mice[16]. In the field, special care has to be taken when vaccinating certain species such as goats and llamas, which appear to be more susceptible to ill effects from the vaccine than other domestic herbivores.

HUMAN VACCINES

In the USSR, a live spore vaccine, reported to be a Sterne strain vaccine[17], is produced for human use by the Tblisi Scientific Research Institute of Vaccines and Serums, 3 Gotu Street, 380042, Tblisi, USSR. The vaccine is administered by scarification of the skin of the shoulder through a 10–20 μl drop of vaccine containing $\pm 4 \times 10^8$ spores. There are, however, numerous patient conditions in which it is recognized in the manufacturer's recommendations that the use of this vaccine is contraindicated.

In the western world, the live spore vaccine is considered unsuitable for administration to humans and vaccines developed for this purpose in the UK in the 1950s and in the US in the 1960s remain in use today. The UK vaccine (licence no. 1511/0037, Department of Health, 10 Russell Square, London WC1B 5EB) consists of an alum-precipitated cell-free filtrate of Sterne strain cultures grown so as to maximize the protective antigen (PA) content. The US vaccine (product licence no. 99, Bureau of Laboratories, Michigan Department of Public Health, Lansing, MI, USA) is an aluminium hydroxide-adsorbed cell-free filtrate of cultures of a non-capsulating, non-proteolytic derivative of strain V770 from a case of bovine anthrax in Florida in 1951[18,19]. This strain is alleged to produce PA in the relative absence of other anthrax toxin components, the lethal factor (LF) and oedema factor (EF), and culture conditions are aimed at further reduction of any LF and EF. Recent serological studies have supplied evidence that the PA content is higher and the LF and EF content is much lower in the US vaccine than in the UK vaccine[14,20] (*Table 1*; also see below).

Table 1 PA, LF and EF contents of existing licensed UK and US human vaccines[a]

Direct measurement – UK vaccine (alum precipitated)
PA = ± 5 μg/0.5 ml dose[22]
LF = ± 2.5 μg/0.5 ml dose[b]
EF = trace[b]

Indirect measurement – antibody titres in human vaccinees and immunized guinea pigs (mean values of accumulated readings detailed in references 14, 20 and 39)

Vaccine[c]		Titres		
		Anti-PA	Anti-LF	Anti-EF
Humans	UK	1024	1024	w[d]
	US	8192	Neg.	Neg.
Guinea-pigs	UK	16400	8192	512
	US	32800	16	Neg.

[a]With the UK vaccine, direct measurement was possible since the alum could be dissolved out with 0.05 M citric acid and the values verified by comparison with pre-precipitated vaccine (the UK vaccine is made in the PHLS Centre for Applied Microbiology and Research for the Department of Health). With the US vaccine (aluminium hydroxide adsorbed), no way was found of dissolving off the aluminium hydroxide or of otherwise separating it from the antigen; consequently, the PA, LF and EF content can only be inferred indirectly by comparison of antibody titres in immunized humans or animals.
[b]Turnbull, unpublished data
[c]All 2 weeks after a course of three bi-weekly doses (0.5 ml in humans, 0.25 ml in guinea pigs).
[d]Weak reaction; below level at which a titre could be assigned

EFFECTIVENESS AND IMPACT OF ANIMAL AND HUMAN VACCINES

As reviewed by Sterne *et al.*[8] the many trials on Carbozoo and other saponin-suspended live spore vaccines in herds, many of them large, of cattle, sheep and horses demonstrated almost dramatically the protective efficacy of these vaccines; marked reduction or complete absence of cases was reported in herds that hitherto had suffered major losses from anthrax. Trials with his own vaccine[9,12]

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on the impact of the spore vaccines in South Africa over the period 1925 to 1941 highlighted the effectiveness of his vaccine. The reduction in catastrophic outbreaks of earlier years brought about by the forerunners to Sterne's vaccine made farmers more critical of the reactions caused by the vaccines. Attempts to reduce these led to a loss of potency and the resulting upsurge of outbreaks in 1934–36. Following replacement of the old vaccines with his new $34F_2$ vaccine, the downward trend commenced again. Now, as then, farmers and veterinarians will testify that the vaccine will effectively cut short outbreaks.

It is much harder to produce data demonstrating and quantifying the effectiveness of the human vaccines. In this country notifications of human anthrax were already on the decline at the time of introduction of the vaccine (*Figure 2*), presumably due to improved factory hygiene and monitoring with, where appropriate, sterilization of imported animal products.

At a time when the US vaccine was being introduced into factories concerned with processing animal products, Brachman *et al.*[21] found it possible to compare case rates among workers at risk in four mills who had received the vaccine and those that had not. Of 26 cases, occurring over a 4-year period, only one occurred in the group that had received the full course of the vaccine; four cases occurred among those who had received only part of the vaccine course and the remainder in those that had received no vaccine. The data indicated a 92.5% degree of effectiveness.

The study of Brachman *et al.*[21] remains the only one supplying hard data on the effectiveness of the vaccines in humans. However, with all the usual cautions that must be applied when extrapolating data from animals to humans, tests in animals have indicated that the protective efficacies of both the UK and the US vaccines are less than ideal (*Table 2*)[13,20,22,23]. The differences observed in the different studies summarized in *Table 2* are attributable to challenge strain differences, the use of different batches of US vaccine and differences in the ages of the guinea-pigs used, but all serve to underscore the need for improved performances.

RESEARCH INTO NEW VACCINES

Overall satisfaction with the performance of the veterinary live spore vaccine has meant that recently the predominant emphasis has been on improving the human vaccines. In addition to the uncertain performance outlined above, the injection into human beings of crude and undefined preparations is increasingly regarded as unsatisfactory, particularly, as in the case of the anthrax vaccines, when they are associated with frequent complaints of unpleasant side-reactions.

It is for these reasons that, over the past few years, a significant amount of work has gone into developing a second generation anthrax vaccine for administration to humans which, as well as being fully defined (containing only essential ingredients and producing effective levels of protection with a single or, at worst, two doses), produces no side-reactions.

Anthrax having become a rare human disease in the west since the 1950s and 1960s, demand for the vaccines had become minimal and for a long period there was little incentive to produce alternatives to those available. Ironically, the incentive that arose was the supposed threat suggested by the belief of western intelligence that a substantial human epidemic of anthrax in 1979 in Sverdlovsk, an industrial city in the Urals, was due to accidental escape of *B. anthracis* from a military biological installation[24]. The real extent and cause of the outbreak in Sverdlovsk remain unrevealed but the result was that the 1980s were a decade of relative opulence for anthrax research, out of which arose major new levels of understanding of the biochemical, molecular and genetic basis of the disease and the host's defences against it.

As far as improved vaccines were concerned, the first of these advances was the ability fully to purify and define the anthrax toxin components, PA, LF and EF, which had first been separated and partially characterized in the 1950s and 1960s[25,26]. The improved purifications[27–31] allowed the development of enzyme immunoassays by which it became possible for the first time to monitor the response to the vaccines in humans and guinea-pigs and to relate the response in the guinea-pigs to protective immunity[14,15,20,22,23,32].

The second major advance with significant bearing on development of improved vaccines was the discovery[33–36] that the genes encoding the toxin lay on a large plasmid subsequently designated pXO1 and that curing *B. anthracis* of this plasmid resulted in non-toxigenic avirulent derivatives.

During the course of vaccine-related studies in the 1980s, these two developments – a sensitive and specific immunoassay for the toxin components and the ability to produce non-toxigenic $pXO1^-$ strains of *B. anthracis* – led to the clarification of a number of matters.

1 It was readily demonstrated that the toxin, or some part of it, was needed in a vaccine for induction of protective immunity. Live non-toxigenic strains conferred no protection while toxigenic non-capsulated (i.e. avirulent) strains, e.g. the Sterne strain, induced good protection[22].
2 The belief, first formulated in 1963[26], that PA (then called Factor II) was the main immunogen has been verified. PA in the absence of EF and LF has now been shown to be capable of producing effective protection both as a purified entity[23,32,37] and when produced free of EF and LF in appropriately cloned *B. subtilis*[15,38]. Consequently, protection has been noted in vaccinated animals with high levels of anti-PA in the absence or near absence of anti-EF

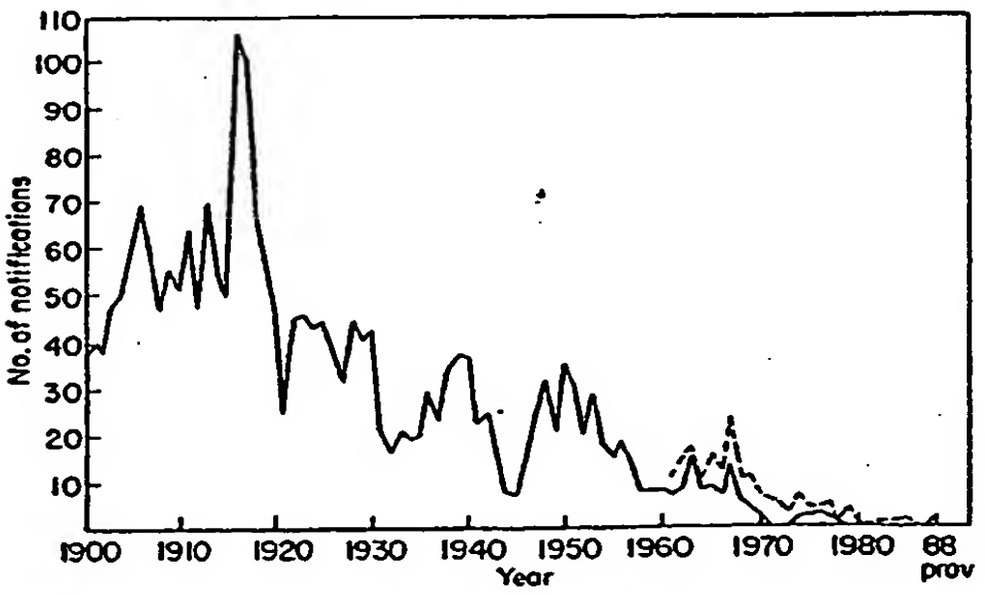

Figure 2 Notifications of human anthrax under the Factories Acts and Public Health Act United Kingdom, 1900–1988 (reproduced from Turnbull *et al.*[35], kindly updated by the PHLS Communicable Disease Surveillance Centre ——, Factories Acts; —·—, Public Health Act)

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Table 2 Protection induced by three doses[a] of the UK and US human vaccines and by one to three doses of live spore vaccines (LSV and ST1) in guinea-pig protection tests

Reference	Vaccine	Challenge strains[b]	Survival (%)		Mean[c] anti-PA titre
			Vaccinated	Unvaccinated controls	
13	US	Vollum/Vollum 1B	100	16	10000
	LSV-3		100		3000
	US	9 other strains	28	3	(10000)
	LSV-3		97		(3000)
15	US	Ames	75	0	20535
	LSV-1		73		392
	LSV-2		88		10392
20	UK	Vollum	100	0	16400
	US		100		32800
	LSV-1		100		4096
	ST1		100		4096
	UK	Ames/NH/Pen Res	33	0	(16400)
	US		17		(32800)
	LSV-1		65		(4096)
	ST1		72		(4096)
	UK	Ames/NH/Pen Res	12[d]	0	(16400)
	US		4[d]		(32800)
22	US	Vollum 1B	71	0	64508
	LSV-2		100		16124
23	US	Vollum 1B	67	0	58310
	LSV-3		87		14404
	US		67	10	31623
	LSV-3		90		2512

[a]The standard vaccination schedule with the human vaccines begins with a short course of three doses 2–3 weeks apart. Full designations of the UK and US human vaccines are in the text. LSV-1=live spore (Sterne strain $34F_2$ livestock) vaccine, one dose ($1–5 \times 10^8$ spores/dose). LSV-2=two doses 2–3 weeks apart, etc. ST1=live spore vaccine made from the Russian vaccine strain. Braces indicate tests carried out simultaneously.
[b]Vollum used to be termed 'vaccine sensitive' and strains such as Ames, NH (New Hampshire) and Pen Res (a naturally penicillin-resistant isolate) were termed 'vaccine resistant' according to the results of the challenge tests in vaccinated animals. Tests with newer vaccine formulations have shown that the latter are not truly resistant (see also *Table 3*).
[c]Note the poor correlation between anti-PA titre and protection. PA is essential to protection but it is not possible to determine the protected status of the individual from that individual's anti-PA titre. Parentheses indicate these figures are a repeat of those above.
[d]Accumulated results of several experiments

and anti-LF[14,20,32]. EF and LF appear capable of inducing only relatively low degrees of protection[23].

3 Although it became apparent that the presence of PA in a vaccine is essential to protection, it is also clear that the relationship between PA and protection is not straightforward (*Table 2*). One or two doses of live spore vaccine may result in better protection but markedly lower anti-PA titres than three doses of human vaccine or even purified PA[20,22,23,39]. At first it was thought that this indicated that one or more other unidentified antigens were necessary for effective protection but further trials and analysis suggested that the phenomenon could be attributed to the involvement of cell-mediated immunity (CMI). The addition of totally non-specific cellular entities such as killed *Corynebacterium ovis*, *Bordetella pertussis* or Freund's complete adjuvant to either the human vaccines or purified PA enhanced their protective effects[20,32]. This stimulation of CMI is more than simply the effect of inciting the coincidental inflammatory response observed by Sterne[40] on combining the live spore vaccine with saponin; the performance of purified PA in inducing protective immunity was not found to be enhanced by irritants such as saponin[20].

These progressive discoveries, then, pointed the way to a number of approaches aimed at second generation improved human vaccines. The experimental vaccines resulting have taken three forms:

1 Purified PA vaccines in which additions aimed at promoting the necessary cellular immune response have been combined with purified PA[20,32,37].
2 Recombinant vaccines, the chief example of which to date has been *B. subtilis* cloned with the PA gene[15,38]. The PA gene has also been cloned into and expressed by baculovirus and vaccinia virus[41], but the results of protection tests with the recombinant viruses have not been published.
3 Mutant vaccines; Ivins *et al.*[42] derived two *Aro*$^-$ mutants by Tn916 mutagenesis of a Sterne strain descendant of *B. anthracis*. Unable to synthesize aromatic amino acids not available in the mammalian host, these would be expected to lack even the residual virulence of the parent pXO1$^+$/pXO2$^-$ Sterne strain and would, therefore, be theoretically safe for use in a live spore human vaccine. Following the discovery by Leppla *et al.*[28,30] that an essential prerequisite for toxic action by anthrax toxin is binding of LF or EF (competitively) to PA before reaching or at the eukaryotic cell surface and that the binding site for LF and EF on PA must first be exposed through cleavage by a trypsin-like protease[43–45], Singh *et al.*[46] identified and deleted the enzyme cleavage site thereby rendering the

Table 3 Protection conferred by experimental alternative anthrax vaccines

Vaccine					
Type	Details	No. of doses	Challenge strain	Protection (% survival)	Reference
Subunit	PA (70 μg) + TriMix[a]	1 or 2	Ames	100	37
	PA (12.5–50 μg) + TriMix or DeTox	1	New Hampshire	75–100	[b]
	as above	2	New Hampshire	100	[b]
Recombinant	10^9 *B. subtilis* cloned with the PA gene	1	Ames	0–6	37
	10^9 as above	2	Ames	6–71	37
	10^9 as above	2	Ames	95	37
	10^{10} as above	2	Ames	100	37
Auxotrophic mutants	10^9 Transposon *Tn916* mutagenized *Aro*–	1	Ames	87–100	37
	10^9 Sterne strain vegetative cells	2	Ames	100	37

[a]Ribi Immunochem Research, Montana, USA. DeTox is a mixture of monophosphoryl lipid A (detoxified endotoxin) and cell wall skeleton from the BCG strain of the tubercle bacillus; TriMix is DeTox + trehalose dimycolate (purified cord factor from mycobacteria)
[b]Turnbull, unpublished results

PA+LF and PA+EF combinations non-toxic (because LF and EF could no longer bind to the PA) without altering the immunogenicity. A Sterne-type strain of *B. anthracis* in which the gene for PA carried the deletion could again be expected to be entirely avirulent.

Table 3 summarizes the prototype vaccines for the immediate future. A combination of PA with adjuvants 'DeTox' or 'Tri-Mix' (Ribi Immunochem Research, USA) perhaps shows most promise for human vaccine purposes among those within these alternatives that have been subjected to protection tests. DeTox is a mixture of monophosphoryl lipid A and cell wall skeleton from the BCG strain of the tubercle bacillus; TriMix is DeTox + trehalose dimycolate. As well as being a chemically defined formulation lacking whole cell bacteria, it has now been shown that considerably better protection can be induced with single doses of these formulations than with even three doses of the conventional UK or US vaccines (see Ref. 38, also Turnbull, unpublished results). This vaccine is essentially ready for clinical trials although a source of funding to cover this complex process[47] has not been identified yet.

The *B. subtilis* recombinant vaccines of Ivins *et al.*[15,38] were able to produce levels of protection in guinea-pigs broadly equivalent to those induced by the conventional Sterne strain live spore vaccine but only when administered in 10- to 100-fold higher doses than the Sterne strain vaccine[15]. The high doses required and the fact that *B. subtilis* produces a host of unspecified enzymes makes this particular vaccine one of simply academic interest, but the real appeal of the recombinant approach is that, with more appropriate bacterial vectors such as *Salmonella* species, it offers the potential for development of oral vaccines for both humans and animals. Furthermore, the recombinant strains could carry immunizing antigens for simultaneous vaccination against several diseases. Prototype bivalent *Salmonella typhi* Ty21a vaccines expressing *S. typhi* O and *Shigella sonnei* O antigens[48,49], *S. typhi* O and *Escherichia coli* LT-B[50] or *E. coli* CFA I[51], and *S. typhi* O and *Vibrio cholerae* O[52] antigens have been designed already. Similarly, bivalent vaccine strains of *Salmonella enteritidis* carrying *E. coli* LTB[53] and *Salmonella typhimurium* carrying the gene for an immunizing fraction of tetanus toxin[54] have been constructed. Theoretically, the carrier could code for protective antigens from more than one pathogen in addition to the *S. typhi* itself.

The auxotrophic mutant vaccines of Ivins *et al.*[42] also proved themselves able to confer protection at levels equivalent to their Sterne strain parent[37] and were shown in Sterne strain-susceptible mice to have lost their virulence. However, they suffered the drawbacks of possessing a self-transmitting tetracycline resistance factor and an ability to revert, albeit at a low rate, to the *Aro*$^+$ parent type. A live vaccine in which the *B. anthracis* produces a form of PA which cannot be enzymatically cleaved into active form[46] may become acceptable for human use should particular advantages over a fully defined chemical (PA) vaccine become apparent. It may, for example, prove able to confer stronger protective immunity with a single dose and, even more probable, longer lasting protection.

NEW ANIMAL VACCINES

The excellent performance of the Sterne live spore vaccine over the half-century since it was first produced has meant that there has been little motivation to develop a new successor. However, the residual virulence it retains for certain animal species, the limited duration of protection conferred and the fact that it must be administered by injection make it less than ideal in certain situations. In developing countries, for example, where syringes and needles are in short supply for human use, let alone for animal purposes, this mode of administration places an immediate impedance on anthrax control. This has obvious consequences for any programmes aimed at global control or eradication of the disease.

Similarly in African wildlife, where anthrax competes with poaching and other man-made threats for eliminating the increasingly precious and dwindling herbivore species, annual intramuscular administration of the vaccine is clearly an impractical approach to control.

Interest in new developments in anthrax vaccines is, therefore, arising in the veterinary context also, particularly with respect to the development of a fully avirulent formulation that can be administered by some simple oral route procedure. *B. anthracis* not being an invasive organism, it is unlikely that simple oral route administration of the existing vaccine would lead to development of immunity, although even this has not been examined yet. Either some means of ensuring the establishment of its microinfection in the gastrointestinal tract must be designed or a genetically engineered invasive organism

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such as *S. typhimurium* carrying and expressing the anthrax protective antigen gene must be constructed and a way of safely administering this without loss of potency, operator hazard or environmental damage must be found. An important challenge, therefore, still lies ahead in the field of veterinary anthrax vaccine and there are some moves now to address this.

FURTHER READING

A more comprehensive account of anthrax vaccine development can be found in reference 56.

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Anthrax Spores Make an Essential Contribution to Vaccine Efficacy

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Anthrax is caused by *Bacillus anthracis*, a gram-positive spore-forming bacterium. Septicemia and toxemia rapidly lead to death in infected mammal hosts. Currently used acellular vaccines against anthrax consist of protective antigen (PA), one of the anthrax toxin components. However, in experimental animals such vaccines are less protective than live attenuated strains. Here we demonstrate that the addition of formaldehyde-inactivated spores (FIS) of *B. anthracis* to PA elicits total protection against challenge with virulent *B. anthracis* strains in mice and guinea pigs. The toxin-neutralizing activities of sera from mice immunized with PA alone or PA plus FIS were similar, suggesting that the protection conferred by PA plus FIS was not only a consequence of the humoral response to PA. A PA-deficient challenge strain was constructed, and its virulence was due solely to its multiplication. Immunization with FIS alone was sufficient to protect mice partially, and guinea pigs totally, against infection with this strain. This suggests that spore antigens contribute to protection. Guinea pigs and mice had very different susceptibilities to infection with the nontoxigenic strain, highlighting the importance of verifying the pertinence of animal models for evaluating anthrax vaccines.

The disease anthrax is caused by *Bacillus anthracis*, a gram-positive spore-forming bacterium. It affects mammals including humans. After entry into the host, the spores germinate and yield toxin-producing, capsulated bacilli. Toxemia and septicemia rapidly lead to death. The bacilli secrete three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), and these proteins combine to form the lethal (PA plus LF) and edema (PA plus EF) toxins. PA is the common cell binding component and is required for toxin activity (14). Two large virulence plasmids, pXO1 and pXO2, encode toxin production and the formation of a poly-γ-D-glutamic acid capsule, respectively (20, 30). Curing *B. anthracis* wild-type strains of either plasmid attenuates virulence. However, the residual virulence of pXO1$^-$ strains appears to be strongly influenced by the backgrounds of both pXO2 and the chromosome (32, 35). A pXO2$^+$ pXO1$^-$ derivative of the Ames strain, a strain often described as particularly virulent (4, 13), has been shown to be as virulent for mice as the parental strain, due exclusively to bacterial multiplication and associated septicemia (35). Curing *B. anthracis* wild-type strains of pXO2 yields toxigenic, noncapsulated, attenuated strains with vaccinal properties. One such strain, the Sterne strain, is used in the form of a live-spore vaccine for the immunization of animals. Although it performs satisfactorily (7, 26), it has side effects in some species. The recent development of a genetically detoxified Sterne strain derivative, RPLC2, may provide a valuable alternative to the residual virulence of the Sterne strain (2). The live vaccine is not considered suitable for human use, but PA-based cell-free vaccines, prepared from culture supernatants of the Sterne strain, have been licensed (7, 5, 27). Multiple immunizations are required to confer protection, and there are cases of reactogenicity. Recombinant PA can now be produced from various heterologous organisms including *Bacillus subtilis* (11, 17, 34), and the safety and consistency of PA preparations have been improved. Highly purified PA preparations and recombinant PA have been tested in various animal models, including mice, guinea pigs, rabbits, and monkeys (4, 10, 33, 36), in combination with various adjuvants (9, 12). These studies have yielded varied results. They also indicate that PA-based vaccines are less protective than live-spore vaccines against virulent isolates of *B. anthracis* (16, 33). Thus, some strains, for example, the Ames strain, have been termed "vaccine resistant" because full protection in guinea pigs immunized with PA is possible only with adjuvants unsuitable for human vaccines (4, 12, 16). Moreover, several studies illustrate the difficulty of evaluating PA vaccines and establishing a direct correlation between PA-specific antibody titers and protection (9, 12, 26, 28, 29).

In recent years, efforts have been made to improve acellular vaccines. There is evidence that spore antigens present in live-spore vaccines make a contribution to protection (3). Immunization with PA targets toxemia but not septicemia, and it is possible that an immune response to spore antigens would enhance protective efficacy by targeting the early steps of infection. We thus tested the efficacy of a vaccine composed of PA and formaldehyde-inactivated spores (FIS) of a genetically detoxified Sterne strain (RPLC2). Virulent *B. anthracis* strains and two animal models were used: (i) guinea pigs, the animals most commonly used for testing anthrax vaccines (13, 16, 26, 29), and (ii) mice, which are very sensitive to anthrax and particularly difficult to protect (12, 33). Inclusion of FIS in an acellular vaccine resulted in total protection against virulent strains in both animal models, under conditions where PA alone failed. However, the findings for the two animal species differed, indicating the importance of assessing the pertinence of animal models for evaluating anthrax vaccines.

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MATERIALS AND METHODS

Bacterial strains. *B. anthracis* strains were grown in brain heart infusion medium or on CAP agar plates (25). Spectinomycin (60 μg/ml) was added as

TABLE 1. Protective immunity induced in guinea pigs by the combination PA plus FIS

Immunization	Protection[a]	Serological response[b] (titer)	
		Anti-PA	Anti-spores
PA	2/9 (22)	9,600	ND
PA + 10^5 FIS	2/4 (50)	17,700	1,000
PA + 10^6 FIS	3/5 (60)	7,900	2,000
PA + 10^7 FIS	5/5 (100)	12,800	2,000
PA + 10^8 FIS	9/9 (100)	11,200	15,000
10^8 FIS	1/4 (25)	<100	11,000
Control	0/10 (0)	<10	<100

[a] Number surviving/number challenged (percent). Animals were challenged with 300 LD_{50}s.

[b] Titers of antibodies against PA and spores were determined by ELISA. Values given are reciprocal geometric mean titers. ND, not determined.

appropriate. The following *B. anthracis* strains were used: the Sterne strain 7702 (pXO1$^+$), its derivatives RPLC2 (carrying point mutations affecting the catalytic sites of EF and LF) (2) and SM11 (with the genes encoding the S layer deleted) (19), and the virulent strains (pXO1$^+$ pXO2$^+$) 17JB (30) and 9602 (a strain isolated in a fatal human case of anthrax in France [1]). Spores of *B. anthracis* strains were prepared as previously described (21). When necessary, spores were inactivated with formaldehyde (4%) after overnight incubation at 37°C. Spores were conserved in sterile water at 4°C.

Construction of strain 9602P. The *pagA* gene carried by pACP41 (22) was cleaved with *NcoI*, blunted, and ligated to the nonpolar spectinomycin resistance cassette (Spc-H+1) (18). The inactivated gene was inserted into pAT113, and the construct was transferred into strain 9602 by "heterogramic" mating as previously described (21). The mutant strain carrying the *pagA* gene deletion (9602P) in place of the wild-type copy was selected on CAP plates containing bicarbonate and spectinomycin. The plates were incubated at 37°C in a 5% CO_2 incubator to allow transcription of the spectinomycin resistance cassette from the *pagA* gene promoter. The construct in 9602P was verified by PCR, and the absence of PA production was checked by immunoblotting using antibodies specific for PA.

Immunization and challenge of experimental animals. Seven-week-old female Swiss outbred mice (six per group) (Iffa Credo, l'Arbresle, France) and female Hartley guinea pigs weighing 200 to 300 g (Charles River, Saint-Aubin les Elbeuf, France) were used for virulence and immunization experiments. The components used for immunization were purified PA (10 μg/mouse and 40 μg/guinea pig) and/or FIS of RPLC2. Aluminum hydroxide (0.3%), which is the adjuvant licensed for human vaccines (5, 27), was used for all immunizations. Mice were injected subcutaneously (200 μl), and guinea pigs were injected intradermally (100 μl). Animals were immunized twice, on days 0 and 15. Serum samples were taken from the retro-orbital plexus of mice and by cardiac puncture from guinea pigs on day 33. Animals were challenged subcutaneously on day 35. The 50% lethal dose (LD_{50}; the dose of spores killing half the animals) of 17JB was 500 spores for mice; the LD_{50} of both 9602 and 9602P was 50 spores for mice. The LD_{50} of 9602 was 100 spores for guinea pigs. Mice were challenged with a dose equivalent to 30 times the LD_{50} of 17JB, 9602, or 9602P, and guinea pigs were challenged with 300 times the LD_{50} of 9602. Surviving animals were sacrificed 2 weeks after challenge.

Serological tests. Enzyme-linked immunosorbent assays (ELISAs) were used to determine titers of antibodies (total immunoglobulin) specific for the purified PA as previously described (23). Titers of antibodies to spore surface proteins were also determined by ELISA. Wells of 96-well microtiter plates (Nunc) were coated with formaldehyde-treated SM11 spores (10^7/well) overnight at 37°C. Spores were then fixed with paraformaldehyde (3.4%). Anti-species antibodies coupled to peroxidase were used at a dilution of 1/1,000. An arbitrary A_{492} value of 0.5 was used to calculate the endpoint titers.

Neutralizing assays on macrophages. Murine macrophages (RAW264.7) were seeded in a 96-well microtiter plate (Nunc) (2×10^4 cells/well) and incubated for 16 h at 37°C under a 5% CO_2 atmosphere. PA (50 ng/ml, the 100% effective concentration) was preincubated for 1 h at 37°C under a 5% CO_2 atmosphere with dilutions of sera from mice immunized with PA alone or with PA plus FIS. The complexes were then incubated with LF (1 μg/ml) and the macrophages for 3 h at 37°C under a 5% CO_2 atmosphere. Cell viability was quantified by a colorimetric assay (8).

Statistics. The χ^2 test was used for statistical analysis.

RESULTS

Protective efficacy of PA plus FIS in guinea pigs. The contribution of FIS to PA vaccine efficacy was first evaluated in guinea pigs (Table 1). The highly virulent strain 9602, which has an LD_{50} similar to that of the Ames strain (13), was used for challenge. Animals receiving PA alone were poorly protected (22%), in agreement with the findings of other immunoprotection studies. In contrast, inclusion of FIS in the vaccine enhanced protection and did so in a dose-dependent manner. Indeed, protection was 100% when 10^7 spores or more were included. Immunization with spores alone, even at the highest dose, provided only partial protection (25%). The difference in survival between guinea pigs immunized with PA alone and those immunized with PA plus 10^8 FIS was statistically significant ($P < 0.05$). The difference in survival between guinea pigs immunized with 10^8 FIS alone and those immunized with PA plus 10^8 FIS was also statistically significant ($P < 0.02$). Thus, the combination PA plus FIS provided full protection under conditions where PA alone or FIS alone failed to do so.

Protective efficacy of PA plus FIS in mice. Two challenge strains, the laboratory strain 17JB and 9602, were used with mice. Their LD_{50}s were 500 and 50 spores per mouse, respectively. Fifty percent and 33% of mice immunized with preparations of PA or FIS alone, respectively, were protected against 17JB (Table 2). In contrast, 100% of those immunized with the combination PA plus FIS survived the lethal challenge, although small numbers of animals were used. In the experiment with strain 9602, using larger number of animals, none of the animals immunized with either PA or FIS alone survived the lethal challenge, confirming the virulence of strain 9602 and the difficulty of protecting mice. Nevertheless, all animals immunized with the combination PA plus FIS were protected against 9602 ($P < 0.01$).

Characterization of the humoral immune response. In both animal models, the immunization procedure elicited significant titers of antibody against PA (>5,000) and spore antigens (>1,000) (Tables 1 and 2). Sera from immunized animals were used to probe blots of spore surface antigen preparations. Few protein bands were recognized (data not shown). The most strongly labeled antigen was a polypeptide with a molecular size above 250 kDa.

TABLE 2. Protective immunity induced in mice by the combination PA plus FIS

Immunization	Protection[a]		Serological response[b] (titer)		
	17JB	9602	Anti-PA	Neutralizing titer[c] ± SD	Anti-spores
PA	3/6 (50)	0/18 (0)	8,600	1,060 ± 300	ND
10^8 FIS	2/6 (33)	0/12 (0)	40	ND	18,000
PA + 10^8 FIS	6/6 (100)	24/24 (100)	12,000	580 ± 120	4,400
Control	0/6 (0)	0/18 (0)	<10	ND	<100

[a] Number surviving/number challenged (percent). Animals were challenged with 30 LD_{50}s of one of the two virulent strains.

[b] Titers of antibodies were determined by ELISA. Values given are reciprocal geometric mean titers. ND, not determined.

[c] Reciprocal of the dilution of the pooled sera sufficient to neutralize half of the activity against macrophages of the lethal toxin. Experiments were performed in triplicate.

TABLE 3. Protective efficacy of FIS against challenge with strain 9602P

Immunization	Protection[a]	
	Guinea pigs	Mice
PA	0/5 (0)	0/6 (0)
10^8 FIS	5/5 (100)	3/6 (50)
Control	0/5 (0)	0/6 (0)

[a] Number surviving/number challenged (percent). Mice were challenged with 30 LD_{50}s, and guinea pigs were challenged with a lethal dose (4×10^8 spores).

To determine if the 100% protection observed with the combination PA plus FIS was the result of higher PA-neutralizing antibody titers, we determined the neutralizing activities of pools of sera from mice immunized with PA alone or with PA plus FIS (Table 2). The toxin-neutralizing activity of the PA-plus-FIS pool was no higher than that of the PA pool. Therefore, the differences in protection observed could not be due to differences in toxin inactivation.

Protective efficacy of FIS against a capsulated PA-deficient strain. Infection involves both spore germination and subsequent vegetative-cell multiplication. The protection conferred by immunization with inactivated spores may act on the first of these processes. To test this, we constructed a challenge strain the virulence of which is entirely due to its multiplication properties. To avoid differences in the virulence background of the strain, we constructed an isogenic nontoxigenic derivative of 9602 (9602P). To make as small a change as possible, we introduced a nonpolar deletion into the *pagA* gene, which encodes PA.

Strain 9602P was highly attenuated for virulence in guinea pigs; 10^6 to 10^7 spores were required to kill half of the animals. As expected, immunization with PA was unable to protect against lethal challenge with 9602P, and no survival was observed (Table 3). In contrast, immunization with FIS gave 100% protection. The difference in survival between the two groups was statistically significant ($P < 0.01$). These data strongly suggest that in guinea pigs, the immune response against FIS is sufficient to protect against infection with 9602P.

Unlike guinea pigs, mice were highly sensitive to 9602P, and the LD_{50} of this strain was similar to that of the wild-type parental strain, 9602 (50 spores per mouse). Thus, although 9602P did not cause toxemia, its overall virulence in mice was not substantially affected. Immunization with PA did not protect mice against challenge with 9602P (Table 3), whereas immunization with FIS protected 50% of the mice against such a challenge. These data point to the higher sensitivity of mice to infection.

DISCUSSION

Anthrax involves both toxemia and septicemia leading to death of the infected host. PA-based vaccines only target toxemia, whereas live-spore vaccines may protect against both the effects. Numerous studies evidence the substantial contribution of PA as a vaccine component, but optimal protection is nevertheless best achieved by live vaccines. Here, we report the successful immunization of guinea pigs and mice with a combination of PA and FIS. In both animal models, protection reached 100%. In the sensitive mouse model, the PA-neutralizing antibody titers, which have been considered a marker of protective immunity (24), were not affected upon addition of FIS. However, protection was 0% after immunization with PA and 100% after immunization with PA plus FIS in mice infected with strain 9602. The effect of spores on protective immunity is therefore not a consequence of greater neutralization of toxin activity. Animals also developed an antibody response to spore antigens, as determined by ELISA. The improved protection may be a consequence of the response to these antigens, as has been proposed for live-spore vaccines (3). The contribution of FIS to protection against infection was demonstrated by constructing a PA-deficient strain, 9602P, and using it for challenge. Immunization with FIS was sufficient to protect guinea pigs totally, and mice partially, against infection with the capsulated 9602P strain. Interestingly, the virulence of 9602P in mice and guinea pigs was very different: guinea pigs were relatively resistant to 9602P, and thus the toxin is likely to be the main virulence factor in anthrax-infected guinea pigs. This probably also explains why guinea pigs are easier to protect with PA-based vaccines than mice. Moreover, successful passive protection with anti-PA sera has been reported in guinea pigs (15). In contrast, mice were as sensitive to 9602P as to the wild-type parental strain, 9602, which supports observations made with $pXO2^+$ derivatives of virulent strains (32, 35). Therefore, control of septicemia might differ greatly between host species. Protection of hosts highly susceptible to infection probably requires more than PA-mediated toxin neutralization.

In summary, we present evidence that inclusion of killed spores greatly enhances the protective efficacy of a PA-based vaccine. Immunization with FIS plus PA provides a synergistic protective immunity acting on both toxemia and infection. The immune response induced by FIS may act early by blocking germination, a critical step at the onset of pathogen multiplication (6, 31). The molecular mechanisms of the protective immunity induced by FIS and the putative role of spore antigens need to be further investigated. It is clear that full protection against anthrax requires a multifactorial immune response. The results presented here may serve as the basis for the first design, for human use, of a subunit vaccine as protective as the current live veterinary vaccine.

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